# Molecular Mapping Genes Conditioning Reduced Palmitic Acid Content in N87-2122-4 Soybean

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## ABSTRACT

**Palmitic acid is one of the two major saturated fatty acids of soybean [*Glycine max* (L.) Merr.] oil that is closely related to nutritional quality of soybean oil. Reduction of palmitic acid content would lower the total saturated fatty acid content of soybean oil and improve the oil quality for human consumption. Several mutant lines with reduced palmitic acid content have been developed in which the genes conditioning palmitic acid content are located at different loci. The objective of this research was to map the genes conferring reduced palmitic acid from N87-2122-4 on the public soybean genetic linkage map with simple sequence repeat (SSR) markers. Four near-isogenic lines with normal and reduced palmitic acid content and the $F_2$ and $F_{2:3}$ generations of a population derived from the cross of 'Cook' × N87-2122-4 were used to perform the SSR mapping of the genes conditioning reduced palmitic acid. The results indicated that a major gene with an allele for reduced palmitic acid contributed by N87-2122-4 is located near the top of Linkage Group (LG) A1. A SSR marker, Satt684 in that region accounted for 38% of variation in palmitic acid content in the $F_2$ generation and 31% of variation in the $F_{2:3}$ generation. On LG-M, Satt175 accounted for 8% of the variation in the $F_2$ and 9% of the variation in the $F_{2:3}$ generation. This minor gene on LG-M had a significant interaction with the gene on LG-A1 in the $F_2$ generation. When combined in a multiple regression equation, these markers explained 51% of total phenotypic variation for palmitic acid content in the $F_2$ and 43% of the variation in the $F_{2:3}$ generations.**

Soybean oil is an important source of vegetable oil for human food and nonfood applications and accounts for approximately 22% of the world's total edible oil production (Glaudemans et al., 1998). Soybean oil consists mainly of palmitic (C16:0), stearic (C18:0), oleic (C18:1), linoleic (C18:2), and linolenic (C18:3) acid. Palmitic acid is one of the two major saturated fatty acids of soybean oil and is closely related with physical, chemical, and nutritional qualities (Wilson, 1991). The average palmitic acid content in the soybean seed oil of common cultivars is ~120 g $kg^{-1}$ (Erickson et al., 1988a; Fehr et al., 1991; Burton et al., 1994). Reduction of palmitic acid content of soybean oil would lower the total saturated fatty acid content and improve the oil quality for human consumption.

The manipulation of soybean oil quality by altering fatty acid composition is an important breeding objective in the USA (Wilson et al., 1981; Topfer et al., 1995). Soybean lines with reduced palmitic acid content have been developed through chemical mutagenesis, recurrent selection, and hybridization (Erickson et al., 1988a; Bubeck et al., 1989; Wilcox and Cavins, 1990; Burton et al., 1994). Previous studies have shown that reduced palmitic acid was conferred by at least two loci (Erickson et al., 1988a; Fehr et al., 1991; Wilcox et al., 1994), and no maternal effects were observed (Schnebly et al., 1994). The alleles conferring reduced palmitic acid from C1726(*fap*1) and A22(*fap*3) are at independent loci (Erickson et al., 1988b; Schnebly et al., 1994). Allelism studies for the lines C1726, N79-2077-12, and N90-2023 indicated that reduced palmitic acid alleles in N79-2077-12 and N90-2023 segregated independently of the *fap*1 allele in C1726 (Wilcox et al., 1994). However, the genes conditioning palmitic acid in N79-2077-12 and N90-2023 have not been assigned gene symbols. It was reported that the genes conditioning reduced palmitic acid in N87-2122-4 were inherited from N79-2077-12 (Burton et al., 1994; Wilcox et al., 1994), and the reduced palmitic acid content in N87-2122-4 was conditioned by a major gene and a genetic modifier (Rebetzke et al., 1998). N87-2122-4 is an important source of reduced palmitic acid genes being used by soybean breeders. Previous studies indicated that genes modifying the major palmitic acid loci could influence the genetic variation of palmitic acid content by increasing or reducing the palmitic acid content (Horejsi et al., 1994; Rebetzke et al., 1998). Modifier genes have been shown to influence palmitic acid by 2 to 23 g $kg^{-1}$ (Horejsi et al., 1994). Understanding function and genomic location of genetic modifier genes would be useful to breeders in developing effective selection schemes to further reduce or stabilize the palmitic acid content in soybean.

Recent advances in molecular marker technology, especially the development of SSR markers in soybean and an integrated soybean genetic linkage map, have made possible the genetic mapping and dissection of qualitative and quantitative traits in soybean (Cregan et al., 1999). The SSR markers are highly amenable for automation and allele sizing which can provide for their use in high-throughput application and multiple trait selection (Diwan and Cregan, 1997; Mitchell et al., 1997). Using restriction fragment length polymorphism (RFLP) makers, Nickell et al. (1994) mapped *fap*2, an allele conferring elevated palmitic acid content from C1727 on LG-D of the public genetic linkage map (Cregan et al., 1999). Brummer et al. (1995) mapped the *fan* allele controlling reduced linolenic acid from C1640 on LG-B2. With a mapping population formed from *Glycine max* × *Glycine soja* Siebold & Zucc., Diers and Shoemaker (1992) mapped quantitative trait loci (QTL) conditioning five major fatty acids mainly on two linkage groups of the USDA/ISU map using RFLP markers. The objective of this study was to map the

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**Abbreviations:** GLM, general linear model; LG, Linkage group; QTL, quantitative trait loci; RFLPs, restriction fragment length polymorphisms; SSRs, simple sequence repeats.

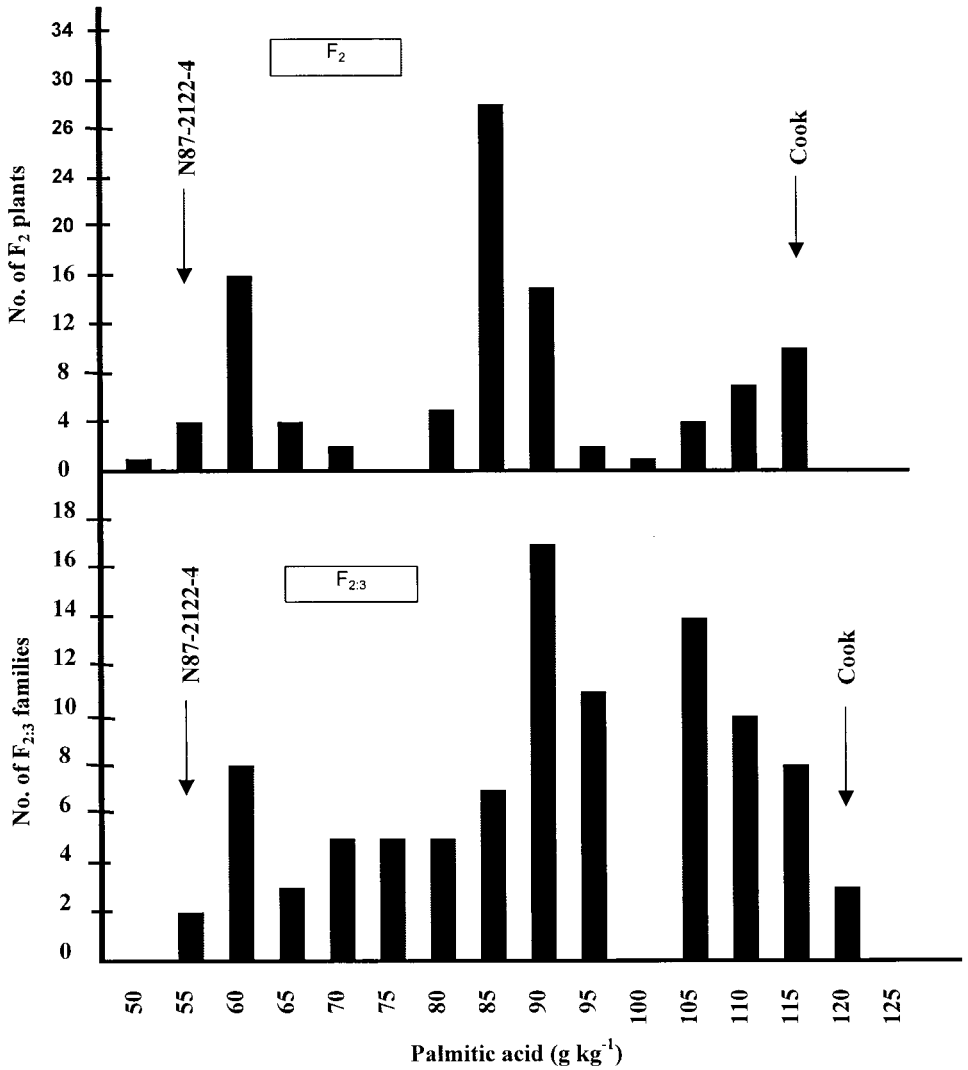


**Fig. 1. Frequency distribution of palmitic acid content in the $F_2$ and $F_{2:3}$ generations of Cook × N87-2122-4 soybean lines.**

genes conferring reduced palmitic acid from N87-2122-4 with SSR markers.

## MATERIALS AND METHODS

Four near-isogenic lines, two with reduced palmitic acid and two with normal palmitic acid content, were derived from 'Benning'[6] × [Cook[3] × N87-2122-4]. These four near-isogenic lines and their parents, Benning, Cook, and N87-2122-4, were used in an initial screening of SSR markers from each linkage group. N87-2122-4 is a source of reduced palmitic acid content (~53 g $kg^{-1}$), while Benning (~123 g $kg^{-1}$) and Cook (~120 g $kg^{-1}$) are cultivars with normal palmitic acid content (Boerma et al., 1992; Boerma et al., 1997; Burton et al., 1994).

A $F_2$ population consisting of 121 plants was derived from the cross of Cook × N87-2122-4. The $F_2$ seeds were harvested from an $F_1$ plant grown in the greenhouse. Each $F_2$ seed was cut into a ¼-seed fragment and ¾-seed fragment with a razor blade. The ¾-seed fragments, containing the embryonic axis, were used for planting, while the ¼-seed fragments along with 10 seeds of each parent were used for fatty acid determination. The $F_2$ seeds along with their parents, Cook and N87-2122-4, were planted in 0.95-L polystyrene cups (two seeds per cup) in the greenhouse. At maturity, each $F_2$ plant was harvested individually. A bulk sample of 12 seeds from each $F_2$ plant and their parents (10 12-seed bulk samples from each parent) were analyzed for fatty acid content in two independent laboratories. If fewer than 24 seeds were produced on an $F_2$ plant, 12-half seed fragments were used for fatty acid analysis in each laboratory.

The seed fatty acid content was analyzed using gas-liquid chromatography of the methyl esters. The analyses were done in the USDA-ARS, Natl. Center for Agric. Utilization Res. at Peoria, IL (USDA-ARS/Peoria) for $F_2$ seeds and in both the USDA-ARS/Peoria and Soybean Research Laboratory (USDA-ARS/North Carolina State Univ.) for $F_{2:3}$ seeds.

DNA was extracted from leaf tissue of a single $F_2$ plant by the modified CTAB procedure of Keim et al. (1988) and diluted to a concentration of 20 ng/μL for the PCR reaction. Leaves from each sample were ground in liquid nitrogen, and 700 μL of CTAB buffer [1.4 *M* NaCl; 100 m*M* Tris pH 8.0; 2% (w/v) CTAB; 20 m*M* EDTA; 0.5% (w/v) Na bisulfate; and 1% (v/v) 2-mercaptoethanol] were added to suspend the powdered materials. The samples were incubated in a water bath at 65°C for 1 h and then 500 μL chloroform/isoamyl alcohol (24:1, v/v) were added. After shaking for 30 min at room temperature, the samples were spun at 13 000 rpm (Beckman Microfuge E, Beckman Instruments, Carlsbad, CA) for 6 min. The supernatant was transferred to a new 1.5-mL tube. Eighty percent volume of isopropyl alcohol was added

to precipitate DNA. The supernatant was decanted and the pellets were washed with 70% (v/v) ethanol. The DNA pellets were then dried and dissolved in 100 μL TE buffer

PCR reactions were prepared on the basis of the protocol by Diwan and Cregan (1997) with slight modifications. The 10-μL reaction mix contained 2 μL of 40 ng template DNA, 1.0× PCR buffer, 2.5 m*M* $MgCl_2$, 100 μ*M* of each dNTP, 0.2 μ*M* each of forward and reverse primers, and 0.5 unit of Taq DNA polymerase. The reactions were performed in a dual 384-well or a 96-well GeneAmp PCR System 9700 Perkin Elmer Applied Biosystems (PE-ABI, Foster City, CA). Fluorescent dye-labeled primers were synthesized by PE-ABI (Foster City, CA). The primers were labeled with 6-FAM, NED, or HEX.

A loading sample for each lane was prepared with 2.5 μL of deionized formamide, 1.5 μL of loading buffer, 0.2 μL of Genescan Rox-500 (PE-ABI, Foster city, CA), and 1 to 3 μL of the pooled PCR products. Genescan ROX-500 is an internal size standard ranging in size from 35 to 500 basepairs. The mixture was denatured at 95°C for 2 min, and approximately 1.0-μL volume was loaded on each of 96 lanes on a 4.8% (w/v) acrylamide: bisacrylamide (19:1) gel with KLOEHN micro syringes (Kloehn Ltd., Las Vegas, NV). Electrophoresis was run with 120-mm well-to-read plate on ABI PRISM 377 DNA Sequencer at 750 V for approximately 1.5 h. Marker data were collected with PE ABI 377-96 DNA Sequencer Collection software. The marker fragments were analyzed with GeneScan and scored with Genotyper software (PE-ABI, Foster City, CA).

The fatty acid data were checked for the distribution and normality by means of SAS programs (SAS Institute, 1989). The broad-sense heritability was calculated on the basis of parent-offspring correlation (Fehr, 1987). The *t* test was used to test the difference in palmitic acid content between two parents and the $\chi^2$ procedure was used to evaluate segregation ratios of marker genotypes (SAS Institute, 1989).

Genetic linkage was estimated by the Kosambi mapping function of MAPMAKER/EXP (Lincoln et al., 1992a). The markers were assigned to linkage groups with the criteria of logarithm of odds (LOD) ≥3.0 and maximum distance ≤37.2 centimorgan (cM) between markers. The order of markers within the linkage group was determined by the 'Compare' command and confirmed by the 'Ripple' command.

The palmitic acid and marker data were analyzed for the presence of QTL. Interval mapping with MAPMAKER/QTL (Lincoln et al., 1992b) was used to estimate the positions of QTL. A minimum LOD score of 2.0 (default) was used for the determination of significance. Single factor analysis of variance (SF-ANOVA) was also used to determine the significance ($P = 0.05$) among SSR genotypic class means by means of General Linear Model (GLM) (SAS Institute, 1989). To detect the epistasis, two-factor ANOVA was performed on all pairs of significant markers. A multiple regression model with a FORWARD option was used for identifying the independent markers linked to the QTL among linkage groups at the 5% significance level.

## RESULTS AND DISCUSSION

The average palmitic acid contents of seed oil from 10 bulk samples of seed were 56 ± 3.5 g $kg^{-1}$ for N87-2122-4 and 117 ± 4.9 g $kg^{-1}$ for Cook in the $F_2$ generation (Fig. 1). On the basis of the *t* test, the palmitic acid content in N87-2122-4 was significantly ($P < 0.05$) lower than that in Cook. The two near-isogenic lines with reduced palmitic acid averaged 60 g $kg^{-1}$ (59 and 60 g $kg^{-1}$), while the two near-isogenic lines with normal palmitic acid averaged 123 g $kg^{-1}$ (123 and 124 g $kg^{-1}$). The range of palmitic acid content in the $F_2$ population was 54 to 123 g $kg^{-1}$ with a mean of 91 g $kg^{-1}$ and standard deviation of 20 g $kg^{-1}$.

Seeds were produced from 98 $F_2$ plants ($F_{2:3}$ lines) and were analyzed for the palmitic acid content to confirm $F_2$ genotypes. The average of palmitic acid of both parents (Cook, 123 ± 4.8 g $kg^{-1}$ and N87-2122-4, 57 ± 8.3 g $kg^{-1}$) in the $F_{2:3}$ generation was similar to that in the $F_2$ (Fig. 1). Means of $F_{2:3}$ lines ranged from 58 to 125 g $kg^{-1}$ with a mean of 94 g $kg^{-1}$ and standard deviation of 17 g $kg^{-1}$. No significant ($P \leq 0.05$) transgressive segregation of palmitic acid content in either the $F_2$ or $F_{2:3}$ was observed. The normality tests of $F_2$ and $F_{2:3}$ populations indicated that the palmitic acid was normally distributed. The broad-sense heritability was 0.94 based on the parent-offspring ($F_2$ and $F_{2:3}$) correlation method. The heritability estimates were consistent with previous reports from Fehr et al. (1991) and Wilcox et al. (1994).

Six markers were selected from each linkage group on the basis of their approximate even distribution across the 20 linkage groups (Cregan et al., 1999). Of these markers, Satt276 on LG-A1 was found to have a common band in the two near-isogenic lines with reduced palmitic acid that differed from a common band in the two near-isogenic lines with normal palmitic acid content.

To search for additional genes conditioning reduced palmitic acid content, three to five SSR markers were initially selected from each linkage group to check the association between SSR markers and palmitic acid QTL in the $F_2$ generation. The markers were chosen to cover the distance less than 40 cM on the public linkage map. On the basis of the SF-ANOVA, three markers, Satt276, Sat_368, and Satt684 on LG-A1 and one marker Satt175 on LG-M, were found to be associated with the variation of palmitic acid content in the $F_2$ and $F_{2:3}$ (Table 1). Linkage maps with additional SSR markers on LG-A1 and LG-M were constructed with MAPMAKER/EXP (Fig. 2 and 3). On the basis of $\chi^2$ tests, all SSR

**Table 1. Soybean SSR markers associated with the genes conferring palmitic acid content in the $F_2$ and $F_{2:3}$ generations of Cool × N87-2122-4.**

| | SF-ANOVA† | | Multiple Reg‡ | | Mean palmitic acid content for marker genotype§ | | |
|---|---|---|---|---|---|---|---|
| Marker/LG | *P* value | $R^2$ | *P* value | $R^2$ | CC¶ | C/N | NN |
| | | % | | % | g $kg^{-1}$ | | |
| **$F_2$ generation** | | | | | | | |
| Satt684/A1 | <0.001 | 38 | <0.001 | 38 | 107 | 92 | 71 |
| Sat_368/A1 | <0.001 | 17 | – | – | 102 | 93 | 79 |
| Satt276/A1 | <0.001 | 14 | – | – | 99 | 94 | 79 |
| Satt175/M | 0.024 | 8 | 0.024 | 2 | 94 | 94 | 80 |
| **$F_{2:3}$ generation** | | | | | | | |
| Satt684/A1 | <0.001 | 33 | <0.01 | 31 | 104 | 96 | 76 |
| Sat_368/A1 | <0.001 | 17 | – | – | 101 | 96 | 83 |
| Satt276/A1 | <0.003 | 12 | – | – | 98 | 96 | 84 |
| Satt175/M | 0.01 | 9 | 0.04 | 3 | 96 | 97 | 83 |

† SF-ANOVA: Single factor analysis of variance.
‡ Multiple Reg: Multiple regression analysis including significant markers ($P < 0.05$) across linkage groups.
§ Marker genotypes for SF-ANOVA.
¶ CC: Homozygous for allele from Cook; NN: Homozygous for allele from N87-2122-4; C/N: Heterozygous for alleles from N87-2122-4 and Cook.

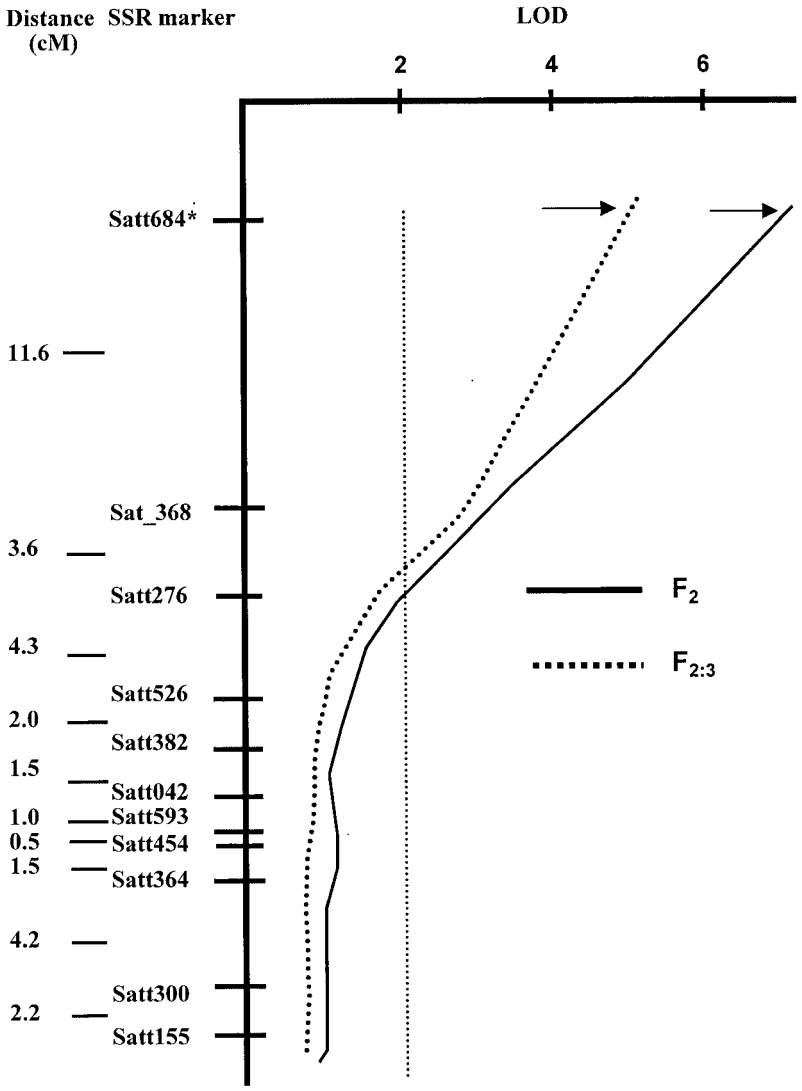


**Fig. 2. Linkage map of Linkage Group A1 and likelihood plot of a major gene conditioning the reduced palmitic acid content in soybean. The arrow indicates the LOD score at Satt684. The QTL conditioning palmitic acid content is most likely distal to Satt684. (*Satt684 is a proprietary SSR marker of the Monsanto Company. Monsanto Company will place the primer sequences for this marker in public domain in December 2001).**

markers segregated in the expected ratio (1:2:1). The order of the markers on both linkage groups is in close agreement with the integrated soybean genetic linkage map (Cregan et al., 1999).

On the basis of the SF-ANOVA of the markers on LG-A1, Satt684 accounted for 38% of total variation, while Sat_368, and Satt276 explained 17 and 14% of variation in the $F_2$ generation (Table 1). Satt175 on LG-M only accounted for 8% of the variation. At both the QTL on LG-A1 and LG-M, the alleles for reduced palmitic acid were contributed by N87-2122-4. Contrast of the three genotypic classes of Satt684 on LG-A1 ($A_1$ $A_1$; $A_1$ $A_2$; $A_2$ $A_2$) indicated that the QTL acted in an additive manner, while the QTL on LG-M showed complete dominance for the normal palmitic acid allele from Cook (Table 1). The data from the $F_{2:3}$ confirmed the results from the $F_2$ generation (Tables 1 and 2).

Although the distribution of $F_2$ palmitic acid phenotypes did not differ significantly ($P \leq 0.05$) from normality, visually it appeared discontinuous. The distribution for the $F_{2:3}$ lines looked continuous and distinct phenotypes could not be clearly determined (Fig. 1). Therefore, the mapping was conducted by means of a quantitative trait approach. Results from MAPMAKER/QTL (Lincoln et al., 1992b) were similar to that found with SF-ANOVA. One major QTL was identified on the top of LG-A1 (LOD = 6.8 and $R^2$ = 38% for $F_2$; LOD = 4.8 and $R^2$ = 31% for $F_{2:3}$) (Fig. 2). Because no polymorphic DNA marker distal to Satt684 was available, it was not possible to determine the precise location or effect of the QTL for the reduced palmitic acid content. The gene action with this marker was additive, which agreed with the results of a classic genetic study (Wilcox et al., 1994). When marker Satt684 was homozygous for the N87-2122-4 allele, the predicted mean for palmitic acid content was 71.4 g $kg^{-1}$. The LOD score of a minor QTL on LG-M, which explained 8% of the phenotypic variation, was very close to 2.0 based on the $F_2$ phenotypes. It exceeded 2.0 and accounted for 12% of variation when based on $F_{2:3}$ phenotypes (Fig. 3). The consistency of $F_2$ and $F_{2:3}$ data analysis supported the QTL locations on both linkage groups.

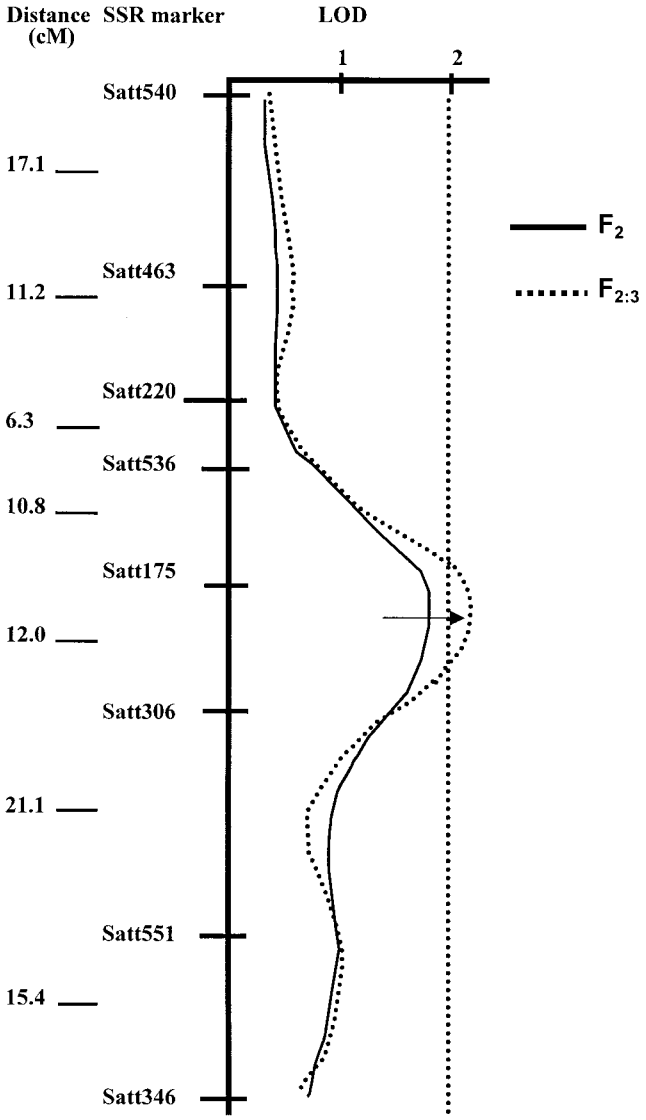


**Fig. 3. Linkage map of Linkage Group M and likelihood plot of a minor gene conferring the reduced palmitic acid content in soybean. The arrow indicates the most likely location of the QTL.**

Multiple regression models are useful in determining the relative importance of the markers associated with the phenotypic variation. The significant markers from LG-A1 and LG-M were included in a multiple regression model. Two markers, Satt684 from LG-A1 and Satt175 from LG-M, were retained in the model in both $F_2$ and $F_{2:3}$ (Table 1). The total variation accounted for by those two markers was 40% in the $F_2$ and 34% in $F_{2:3}$ generations based on the multiple regressions. Although the $R^2$ for Satt175 was small, it significantly ($P < 0.05$) affected the palmitic acid content.

The interaction between markers represents an epistatic effect. To evaluate the importance of epistasis, the six pairs of the four markers detected by SF-ANOVA were tested for the interaction using a two-factor ANOVA (SAS Institute, 1989). The interaction between markers Satt684 and Satt175 was significant ($P = 0.045$) in the $F_2$. The contrast test indicated that the interaction was from a quadratic term of two markers

**Table 2. The effect on palmitic acid content of the interaction between the soybean SSR markers Satt684 (LG-A1) and Satt175 (LG-M) in the $F_2$ and $F_{2:3}$ generations of Cook × N87-2122-4.**

| Satt684 (LG-A1) | Satt175 (LG-M) CC† | C/N | NN | Mean |
|---|---|---|---|---|
| | g kg$^{-1}$ | | | |
| **$F_2$ generation** | | | | |
| CC | 107 | 106 | 107 | 107 |
| C/N | 88 | 99 | 78 | 92 |
| NN | 84 | 71 | 60 | 71 |
| Mean | 94 | 94 | 79 | |
| **$F_{2:3}$ generation** | | | | |
| CC | 104 | 105 | 102 | 104 |
| C/N | 93 | 103 | 82 | 96 |
| NN | 84 | 77 | 68 | 76 |
| Mean | 96 | 97 | 83 | |

† CC: Homozygous for the allele from Cook; NN: Homozygous for the allele from N87-2122-4; C/N: Heterozygous for the alleles from N87-2122-4 and Cook.

(Table 2). The allele from N87-2122-4 on LG-M only affected the variation in palmitic acid in the presence of the allele from N87-2122-4 on LG-A1. When both markers have homozygous alleles from N87-2122-4, palmitic acid was reduced to 60 g kg$^{-1}$, which is near the palmitic acid content of N87-2122-4 (56 g kg$^{-1}$) in the $F_2$ experiment (Table 2). When the interaction term was included in a multiple regression model, two markers Satt684 and Satt175 accounted for 51% of total phenotypic variation in the $F_2$ generation. In the $F_{2:3}$ generation, the interaction between markers Satt684 and Satt175 approached significance ($P = 0.1$). Including the two markers in the regression model accounted for 43% of the variation in the $F_{2:3}$ generation. Rebetzke et al. (1998) reported that one major gene and one modifier gene were responsible for conditioning of reduced palmitic acid content in N87-2122-4. The same phenomenon was also observed in palmitic acid content of soybean lines with *fap1fap1fap3fap3* genotypes (Horejsi et al., 1994) and in the stearic content of soybean lines with major allele *fas* (Lundeen et al., 1987). The result from this study was consistent with the report from Rebetzke et al. (1998).

To simulate an approximate location of the major gene conditioning reduced palmitic acid from N87-2122-4 on LG-A1, three genotypic classes of palmitic acid ($A_1A_1$, $A_1A_2$, $A_2A_2$), were grouped on the basis of the average of both $F_2$ and $F_{2:3}$ data. The palmitic acid content of lines to include in each homozygous genotypic class was defined by the mean of palmitic acid content ±2SD of a parent grown in the same environments. The heterozygous class included lines with palmitic acid contents intermediate to the two homozygous classes. This approach was used by Stoltzfus et al. (2000) in the study of a *fap5* allele. Specifically the families with palmitic acid less than 71 g kg$^{-1}$ were classified as homozygous for the N87-2122-4 allele and greater than 109 g kg$^{-1}$ as homozygous for the Cook allele. All other families (range of 72–108 g kg$^{-1}$) were grouped as being heterozygous. Three genotypic classes of this simulated marker were scored as A (homozygous for Cook allele), B (homozygous for N87-2122-4 allele), and H (heterozygous for Cook and N87-2122-4 alleles) and mapped with the other SSR markers using MAPMAKER/EXP. The

simulated marker mapped 14.5 cM distal to the Satt684 on LG-A1 and accounted for 87% of variation on the basis of the average of $F_2$ and $F_{2:3}$ data. Because of the effect of the minor gene on LG-M and the subjective determination of the phenotypic range to include in each genotypic class, the classification of three simulated marker classes might not be precise. However, this simulated marker could be used as a reference to indicate the approximate location of the major gene on the genomic map. This strategy was previously used by Tamulonis et al. (1997) to locate a soybean resistance gene to southern root-knot nematode [*Meloidogyne incognita* (Kofoid and White) Chitwood], which has recently been verified with newly developed SSR markers (Li et al., 2001). These methods were also used to map a disease resistance gene in pea (*Pisum sativum* L.) and a K+ and Na+ discrimination QTL in wheat (*Triticum aestivum* L.) (Dirlewanger et al., 1994; Dubcovsky et al., 1996). Our results established the presence of a major gene conditioning palmitic acid content on LG-A1 and a minor gene on LG-M. Future efforts will be directed to identify other closely linked markers for the major gene on LG-A1 that will be useful for the marker-assisted selection in a breeding program.

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